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Role of Plant Host in Determining Differential Responses to *Ralstonia solanacearum* and *Glomus mosseae*

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Abstract: A pot study was aimed to investigate the role of tomato in determining differential response to bacterial wilt causal agent *Ralstonia solanacearum* pathogen and arbuscular mycorrhizal fungi (AMF) *Glomus mosseae*. Disease severity was measured after 10, 20 and 30 days of plant growth. The pathogen and dual treatment (*R. solanacearum* with *G. mosseae*) were not significantly different at the end of this experiment. Soil pH was greatly influencing the pathogen and AMF microbe. *Glomus mosseae* mycorrhizosphere was more alkaline (pH 5.9) compared to the pathogen mycorrhizosphere (pH 4.9). The concentration of bacterial cell in the *R. solancearum* soil was not different from the dual treatment after 60 days of plant growth. Spore germination was influenced by the interaction between the soil pathogen and AMF. Spores number in the dual treatment at 60 days was less than the original number added. Root colonization percentage in *G. mosseae* (61%) was significantly more than the dual treatment (16%). This provide an evidence about the role of plant host in increasing the spores germination influenced by many substances produced by the host root (root exudates). The results demonstrated that the role of plant in determination the relationship between soil-borne pathogen and antagonistic microbe was critical.

Key words: Arbuscular mycorrhizal fungi, bacterial wilt, disease severity, root exudates

INTRODUCTION

Under natural condition, 90% of all plant species are estimated to form arbuscular mycorrhizal fungi. It is known to improve plant nutrition like P when plant grown in nutrient poor soil (Smith and Read, 2008). The morphological differential of AM infection structure, only occur in the presence of signals released by host roots, allowing a functional symbiosis to be established (Azcón-Aguilar and Barea, 1995). In spite of this, the process of recognition event by host involve specific regulation of the germination event by host derived signals (Cristiana, 2006). When no host-derived signals from the surrounding environment were perceived by germinating spores, fungal hyphae underwent a programmed growth arrest and resource reallocation, allowing long-term maintenance of viability and host infection capability (Logi *et al*., 1998). Mycorrhizal fungi interact with a wide range of soil organisms in the bulk soil. These interactions may be inhibitory or stimulatory, including, chemical interaction, physical interaction and indirect effect (Fitter and Grabaye, 1994). Mycorrhizal colonization changed the bacterial community structure on the root surface and in the non-rhizosphere soil (Marschner and Baumann, 2003).

Ralstonia solanacearum is a causal agent of bacterial wilt disease for more than 200 plant species worldwide (Vailleau *et al*., 2006). *Ralstonia solanacearum*, a widely distributed and economically important plant pathogen, invades the roots of diverse plant hosts from the soil and aggressively colonizes the xylem vessels, causing a lethal wilting known as bacterial wilt disease (Tans-Kersten *et al*., 2001). This organism is a heterogeneous species with genetic and phenotypic variability (Hayward, 2000). *Ralstonia solanacearum* can survive in the soil for long periods in the absence of host plants (Steck, 2001). The use of antagonistic microorganisms is one of the alternative strategies to control bacterial wilt disease (Wydra *et al*., 2005). Arbuscular mycorrhizal fungi were shown to increase the plants tolerance to soil-borne pathogens (Abdalla and Abdel Fattah, 2000). The AM symbiosis influences the structure and function of surrounding bacterial communities (Marschner *et al*., 2001). It has shown that

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the soil around fungus hyphae supports different bacterial activities and bacterial community composition from those of the mycorrhizo- sphere (Andrade *et al*., 1998). AMF cause qualitative as well as quantitative effects on bacteria have been reported by Vázquez *et al*. (2000). The competition for infection loci was one of the mechanisms involved in which AM fungi inhibited the soil-borne diseases (Vigo *et al*., 2000). Biologically active substances such as amino acids, plant hormones, vitamins and other organic compounds can be produced by soil microorganisms and can stimulate the growth rate of mycorrhizal fungi. Volatile substrate like CO_2 could also be important (Azcón-Aguilar and Barea, 1995).

In view of the earlier, the present study was carried out to determine the interaction between *R. solanacearum* and *Glomus mosseae* in the absence of plant host and to observe the possible role of plant in determining differential response to *R. solanacearum* as a pathogen and *G. mosseae* as a mutualisitic microbe.

MATERIALS AND METHODS

Soil preparation: This study was conducted during August-October 2007. Serdang series soil was collected from the campus of Universiti Putra Malaysia. The soil was sieved using 5 mm pore size sieve and mixed with the sand soil in 3:1 v/v. The soil mixture was autoclaved at 121°C for 1 h twice and 15 PSI. Chemical characteristic of the soil are as follows: pH 5.50, 0.13% N, 0.023% P, 0.30% K, 0.063% Ca, 0.034% Mg, 0.063% S, 1.52 % Fe, 0.0034% Mn, 0.0057% Zn, 0.00064% Mo, 0.0003% B, 0.0015% Cu (Sharifuddin, 1984). Soil mixture was then placed in 20 cm diameter pots.

Root exudates collection: About 20-30 tomato seedlings (14 days old) were rinsed with sterile distilled water several times and placed in flask containing 50 mL sterile distilled water for 24 h. The root solution was pooled by using 0.45 μm sterilized filter. The solution was concentrated 1/10 the original volume by rotary evaporator at 50°C (Karlos and Safir, 1987). It was filtered again and stored at 4°C. The product was checked for saprophyte contamination by using potato dextrose agar (PDA) before use.

Mycorrhizae and *R. solanacearum* preparation: *Glomus mosseae* spores were grown in corn (*Zea mays*) pot culture in glass house for 3 months and stored under laboratory conditions (15-20°C). Wet sieve technique was followed to isolate and purify the AMF spores (Gerdman and Nicolson, 1963). Mature and healthy spores were isolated and collected from the pot culture. Hundred spores/100 g dry soil were poured to the soil and mixed well. Tomato root exudates were used to water the mixture for one week to stimulate *Glomus mosseae* spore germination before any treatment (Shang *et al*., 2000).

Ralstonia solancearum race 2 biovar 3 was re-cultured using CPG media described by Cuppels *et al*. (1978). Suspension of *Ralstonia solancearum* was prepared (1×10^8 cfu) and poured over the soil mixture and was left for 1 week for interaction under glasshouse conditions (25-30±2°C).

Plant growth conditions: Red rock tomato cultivar was used. The seeds were surface sterilized with 90% ethyl alcohol for 15 sec and washed with sterile distilled water and germinated 10 days old, tomato seedling were transplanted (1 seedling per pot) to the pots after 7 days of interaction between AMF spores and the pathogen. The plants were kept under glasshouse conditions with temperature 25-30±2°C and continue light.

Shoot and root biomass: Shoot and root dry weight was measured for all treatments at the end of this experiment. Shoot and root samples were kept in the oven (70°C for 24 h), then the dry weight was measured for each single plant.

Disease progress: The development of disease symptoms was rated using the following scale (0 = no disease symptoms, 1 = 1-25% one or two of plant leaves wilted, 2 = 26-50% all plant leaves wilted except the top 2 or 3 leaves, 3 = 51-75% all plant leaves wilted, 4 = all plant died).

The following formula was adopted to calculate percentage disease severity (Eq. 1):

$$\text{Disease severity (\%)} = \frac{X_1 + X_2 + ... + X_n}{Y \times \text{maximum rating scale}} \times 100 \quad (1)$$

Where:
X = Score of disease severity of each seedlings
Y = Total No. of tested seedling

Soil pH: Soil pH was measured using pH meter for three times, seven days before planting, 30 days after planting (during the interaction between *G. mosseae* and *R. solanacearum*) and 60 days of plant growth (at the end of this experiment).

Population dynamics of *G. mosseae* and *R. solanacearum*: Arbuscular mycorrhizal fungi spores were separated from the soil by wet sieving and decanting technique (Gerdman and Nicolson, 1963). One hundred gram soil were collected and mixed well with water. The mixture was poured through different sieve sizes

(250, 106 and 53 μM). After several time of washing sieves, the liquid was collected in Petri dish and the spores were counted under binocular- microscope.

Bacterial suspension was prepared 10^8 cfu mL^{-1} ($OD_{600\ nm}$ = 0.5) and presented as colony forming unit (CFU) using spectrophotometer three times, 7 days before planting, 30 days after planting (during the interaction between *G. mosseae* and *R. solanacearum*) and 60 days of plant growth.

Mycorrhizal assessments: The percentage of adventitious and lateral root colonization was evaluated microscopically followed by clearing the roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (Phillips and Hayman, 1970). The following formula (Eq. 2) was used to calculate root colonization percentage (Giovannetti and Mosse, 1980):

$$\text{Colonization (\%)} = \frac{\text{No. of colonized segments}}{\text{Total No. of segments examined}} \times 100 \quad (2)$$

Experiment layout: The treatments were arranged in randomized complete design (RCD) with four treatments under glass house condition in the campus of Universiti Putra Malaysia/Malaysia. *R. solanacearum* (RS), *G. mosseae* (GM), *Glomus mosseae* with *R. solanacearum*, Control (C).

Data analysis: Data was subjected to an analysis of variance (Tuky Post Hoc Test) using SPSS 15.0 software (SPSS Inc., Chicago, USA).

RESULTS

Morphology biomass: The shoot dry weight was increased significantly in AMF treatments compared to included with the pathogen. The dual treatment (*G. mosseae* with *R. solanacearum*) and the control treatments were not different statistically in the shoot dry weight. The root dry weight was the lowest in the pathogen treatment and it was not significantly different from varied relative to *G. mosseae* with *R. solanacearum* and control treatment (Table 1). The *G. mosseae* treated roots were the highest in root colonization percentage and it was different in comparison to control plat root. The dual treatment (*G. mosseae* with *R. solanacearum*) and *G. mosseae* treatments were different in root colonization significantly (Table 1).

Spore population: The spore population was less than the original number added after 7 days of the interaction between *G. mosseae* and *R. solanacearum*, different between both treatments was observed. After 30 days of plant growth, the spore's number increased in soil with *G. mosseae* but not in significant level. At 60 days, the spore number increased significantly in *G. mosseae* mycorrhizosphere and it was less than the original number in the dual inoculums treatment (Fig. 1).

Table 1: Effect of *G. mosseae*, *R. solanacearum*, *R. solanacearum* with *G. mosseae* and control on tomato shoot dry weight, root dry weight and root colonization percentage

Treatments	Shoot dry weight (g)	Root dry weight (g)	Root colonization (%)
R. solanacearum	0.78a	0.63a	0.00a
G. mosseae	2.75b	3.25b	61.25b
R. solanacearum with *G. mosseae*	1.54ab	1.29a	16.25c
Control	1.70ab	2.06ab	0.00a

Means in columns followed by the same letter(s) are not significantly different, according to Tukey Post Hoc Test ($p \leq 0.05$)

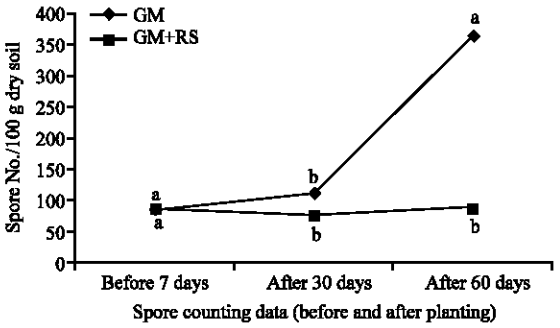

Fig. 1: Spore number of *Glomus mosseae* in soil inoculated with *Glomus mosseae* and *R. solanacearum* within different time period. Means values followed by the same letter(s) are not significantly different according to Tukey Post Hoc Test ($p \leq 0.05$)

Soil pH measurement: After 7 days of seedling transplanted, soil pH was different in *R. solanacearum* treated soil compared to other treatments. The dual treatment (*G. mosseae* with *R. solanacearum*) did not vary significantly in comparison with *G. mosseae* treatment soil, but (*G. mosseae* with *R. solanacearum*) was different compared to control. At the end of this experiment, the dual treatment, (*G. mosseae* with *R. solanacearum*) and control were not different statistically. The soil pH in *R. solanacearum* was the lowest but not different compared to the control and (*G. mosseae* with *R. solanacearum*) treatments (Table 2).

The concentration of the pathogen was different between *R. solanacearum* and *G. mosseae* with *R. solanacearum* before 7 days of plant growth. After 30 days, the concentration of pathogen cell was increased

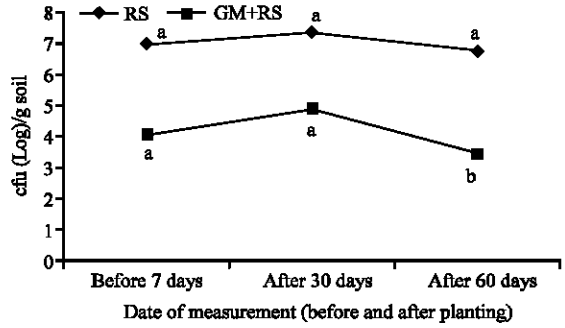

Fig. 2: Effect of different treatments, *Glomus mosseae* (GM) and *R. solanacearum* (RS), within different measurement time on colony forming unit (cfu). Means values followed by the same letter(s) are not significantly different according to Tukey Post Hoc Test ($p \leq 0.05$)

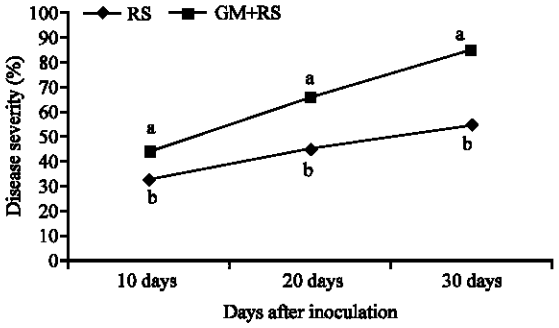

Fig. 3: Disease severity curves of bacterial wilt of tomato inoculated with *R. solanacearum* (RS) and *Glomus mosseae* (GM) during 10 days interval time. Means values followed by the same letter(s) are not significantly different according to Tukey Post Hoc Test ($p \leq 0.05$)

Table 2: Effect of different treatments, *Glomus mosseae*, *R. solanacearum*, *Glomus mosseae* with *R. solanacearum* and control on soil pH within three different times (7 days before plant growth, 30 and 60 days after plant growth

	pH		
Treatments	7 days	30 days	60 days
R. solanacearum	5.03a	5.02a	4.98a
G. mosseae	5.43b	5.60bcd	5.91ab
R. solanacearum with *G. mosseae*	5.52b	5.36ac	5.29ab
Control	5.42b	5.78d	5.50ab

Means values followed by the same letter(s) are not significantly different according to Tukey Post Hoc Test ($p \leq 0.05$)

in both treatments *R. solanacearum* and (*G. mosseae* with *R. solanacearum*) but not in a significant value. The different between pathogen and dual inoculation treatment was clear at the end of this study (Fig. 2).

Disease severity: After 10 days of applying *R. solanacearum*, the plants were stressed because of the drastic effect of infection by the pathogen. The plants inoculated with both *G. mosseae* and *R. solanacearum* were not significantly different when compared to pathogen treatment. Twenty days later, the symptoms were appeared more serious in *R. solanacearum* but it was not different relative to the symptoms recorded in the complex treatment *(G. mosseae* and *R. solanacearum*). After 30 days of pathogen inoculation, the pathogen and dual treatment were not varied significantly (Fig. 3).

Simple regression analysis was used to understand the relationship between spores number and pH soil in different interval time. Small positive relationship was observed ($R^2 = 0.312$) before plant growth and the relation became more weak after 30 days of plant growth ($R^2 = 0.052$). After 60 days of plant growth, moderate relationship was recorded ($R^2 = 0.614$) (Fig. 4).

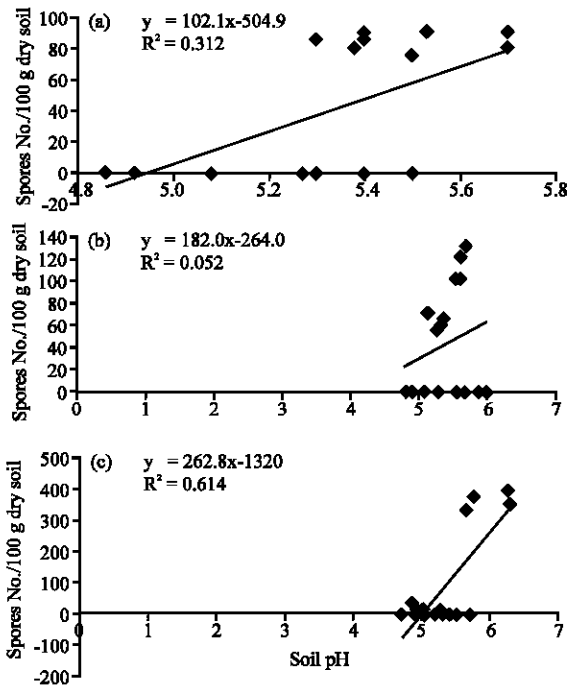

Fig. 4: The relationship between pH and spores No. (a) 7 days before (b) 30 days and (c) 60 days after plant growth

The relationship between bacterial concentration (cfu) and soil pH was detected using the simple regression model. It was clear that negative relationship between cfu and pH during the three period of measurement, but it was small before plant growth and more negative after 30 days of plant growth. After 60 days the relationship was negative (Fig. 5).

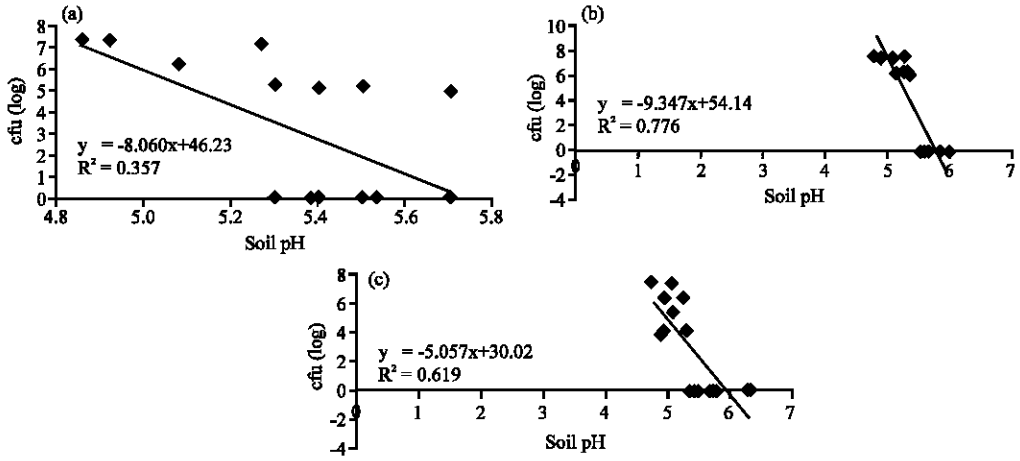

Fig. 5: The relationship between pH and cfu No. (a) 7 days before, (b) 30 days and (c) 60 days after plant growth

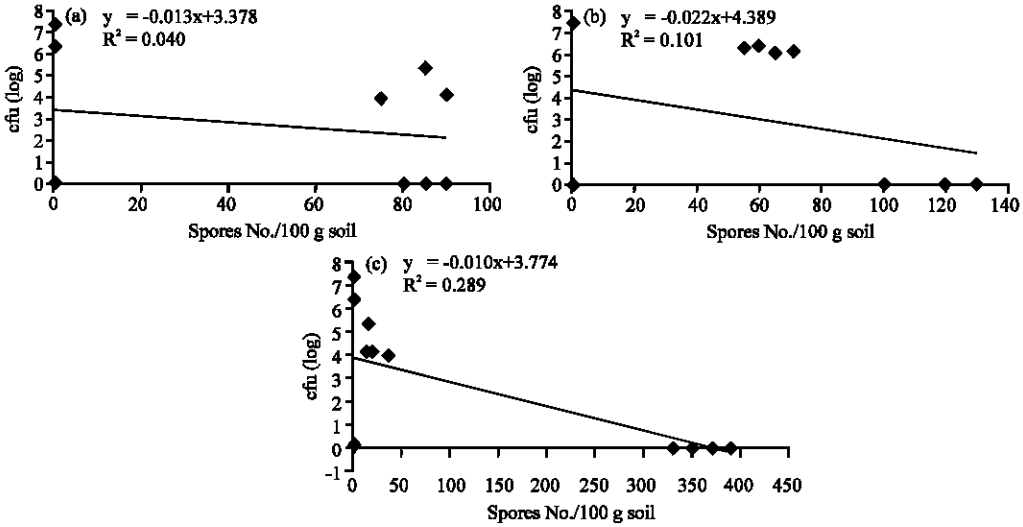

Fig. 6: The relationship between spores No. and *R. solanacearum* concentration (cfu) (a) 7 days before, (b) 30 days and (c) 60 days after plant growth

Negative relationship was observed between spores number and bacterial concentration within three detected time (Fig. 6).

DISCUSSION

Few research were conducted to study the fungal-bacterial pathogen interactions, the reasons for this are unclear but could be perhaps indicate an area that deserves further research in the future (Whipps, 2001). Arbuscular Mycorrhizal (AM) fungi are obligate symbionts that need their plant hosts to complete their life cycle (Smith and Read, 2008). In the absence of the plant, germlings arrest growth after a few days and retract most of their cytoplasm back into the multinuclear spores (Requena *et al*., 2006). The present investigation showed that plant host was an important and critical factor to suppress bacterial infection and increase plant colonization percentage by AMF. *Glomus mosseae* was unable to protect plant against bacterial wilt causal agent related to the low colonization percentage by AMF and this may be due to the inhibition of AMF spores during the direct interaction between AMF and the pathogen (before planting tomato). The same results were obtained by Walley and Germida (1997), they found that *Glomus clarum* spore germination was inhibited as a results of indirect interaction between AMF and *Pseudomonas* species suggested that a nonvolatile, diffusible substance(s) produced by *Pseudomonas* strains may inhibit *G. clarum* spore germination.

In the present study, the spores number in *G. mosseae* treatment were less than the original number used this is due to the dispersal of spores in the soil at the beginning of this experiment. The spores number were decreased in the dual treatment (*G. mosseae* with *R. solanacaerum*) after 30 days of plant growth, this is could be related to the chemicals effect of *R. solanacearum* which can produce some inhibitory substances like bacteriocins. The result in this study was not agree with that found by Araujo *et al.* (2004) they found that some *R. solanacearum* virulent strains can produce potential bacteriocins which can biologically control tomato bacterial wilt *in vitro* conditions. In our finding bacteriocin could be the reason behind the inhibition of spores germination.

The second reason behind the reduction of spores number, perhaps related to the invasion of *G. mosseae* spores by the pathogen for feeding during the absence of host plant. The same results confirmed by Levy *et al.* (2003), they found that 12% of *Giaspora decipiens* were invaded by *Burkholderia vietnamiensis*, in their study, the germination of *Giaspora decipiens* was significantly enhanced by inoculation with *Burkholderia vietnamiensis* but not by inoculation with *Burkholderia pseudomallei*, meaning that bacteria can have either positive or negative effect on germination of spores of AMF.

Mycorrhizal fungi and *R. solanacearum* were computing for root feeding and colonization sites, which can explain the weakness of colonization and root infection by the pathogen. Present results were confirmed by Vigo *et al.* (2000), they reported that the competition for infection loci was one of the mechanisms involved in which AM fungi inhibited the soil-borne diseases) another results documented by Fitter and Grabaye (1994) were illustrated that mycorrhizal fungi compute with rhizbacteria during their pre-symbiotic phase, while growing from the propagate toward the root, which may reduce the development and formation of mycorrhizal fungus.

The interaction between AMF and bacterial wilt pathogen during the absent of plant host resulted in a significant reduction of *R. solanacearum* concentration cell in the soil compared to the pathogen treatment, but the pathogen was more concentrated in the dual treatment after 30 days of plant growth due to the inability of *G. mosseae* to resist the pathogen effect.

Soil pH was a critical factor in determination of the interaction between *G. mosseae* and *R. solanacearum* and studying endomycorrhizal fungi ecology. Soil pH in the pathogen treatment was more acidic compared to *G. mosseae* treatment, which was not far from the initial soil pH. Effects of soil pH on AMF have been reported by several investigators (Wang *et al.*, 1993). Inoculation with *Glomus* sp. increased soil pH (7.0) resulted in more nutrient uptake, finally the plant growth increased. In this study root exudates have been observed to enhance germination of spores of AMF. These results were confirmed by Walker *et al.* (2003), they reported that AMF spore stimulation and germination by root exudates in the absence of other microorganisms. In the current study, the *G. mosseae* spore germination was observed after the using of root exudates for 10 days before any soil treatment. Present finding was agreed with that documented by Douds and Nagahashi (2000) they found that root exudates do appear to play an important role in mycorrhizal formation and spore germination. The growth of *R. solanacearum* was not influenced by the root exudates treatment. The most suppressive compounds effect were glucose, proline, glutamine, serine, arginine and lysine. Biochemical changes associated with plant defense, direct competition or inhibition and of antagonistic microbiota are an important mechanisms to explain the ability of AMF for the suppression the soilborne pathogens (Whipps, 2001). In the current study, the root exudates had an appositive stimulatory effect in spore germination and root colonization in before adding *R. solanacearum* suspension.

The significant reduction of root and shoot dry weight in the dual treatment compared to *G. mosseae* and control treatment indicated that the ability of *G. mosseae* was feeble to build up a strong hyphal net for more nutrient uptake and root strengthen, this probably, due to the pathogen inhibitory effect which resulted in slow plant growth and small root and shoot system. Present results conflict with the finding by Karaginnidis *et al.* (2002), they found that the growth of plants inoculated with AMF and the pathogen were more resistance to the pathogen due to the more hyphal net produced by the AMF which allow the root to absorb more nutrients from the soil. The root colonization was more in *G. mosseae* treatment because of undisturbed conditions compared with the root of dual inoculation treatment which was colonized in a very low percentage, so that the soil conditions was disturbed by the pathogen. Different results were exhibited by Jamil *et al.* (2003), they demonstrated that AMF under semi-arid conditions is a very efficient in establishment plant on disturbed soil. The contest between *G. mosseae* and *R. solanacearum* for infection point considered as a main reason behind the low percentage of root colonization in dual treatment. It was found that AMF spore germination not fully controlled by plants. The same results were reported by Douds and Nagahashi (2000), they reported that the

germination of the spore is not thought to be under direct control of the plant as spores have been germinated under experimental conditions in the absence of plants both *in vitro* and in soil. However, the rate of spore germination can be increased by plant host root exudates.

In conclusion, present data suggested that plant host is the main factor for mycorrhizal fungi spore germination and hyphal development and the interaction between *Glomus mosseae* and bacterial wilt causal agent was controlled and influenced by other factors such as spores number, soil pH, plant age, spore germination ratio, root exudates, bacterial cell concentration, soil macro and micronutrient, and some other environmental conditions.

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